Antimicrobial Agents and Chemotherapy

Genetic Determinants Involved in the Susceptibility of *Pseudomonas aeruginosa* to β-Lactam Antibiotics

Carolina Alvarez-Ortega, Irith Wiegand, Jorge Olivares, Robert E. W. Hancock and José Luis Martínez

Antimicrob. Agents Chemother. **2010, 54(10):4159. DOI: 10.1128/AAC.00257-10.**
Published Ahead of Print 2 August 2010.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2010, p. 4159–4167 Vol. 54, No. 10
0066-4804/10/$12.00 doi:10.1128/AAC.00257-10

Genetic Determinants Involved in the Susceptibility of *Pseudomonas aeruginosa* to β-Lactam Antibiotics▽

Carolina Alvarez-Ortega,[1]† Irith Wiegand,[2]†‡ Jorge Olivares,[1] Robert E. W. Hancock,[2] and José Luis Martínez[1]*

Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Darwin 3, Cantoblanco, Madrid, and CIBERESP, Madrid, Spain,[1] and Centre for Microbial Diseases and Immunity Research, Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4[2]

Received 22 February 2010/Returned for modification 14 May 2010/Accepted 23 July 2010

The resistome of *P. aeruginosa* for three β-lactam antibiotics, namely, ceftazidime, imipenem, and meropenem, was deciphered by screening a comprehensive PA14 mutant library for mutants with increased or reduced susceptibility to these antimicrobials. Confirmation of the phenotypes of all selected mutants was performed by Etest. Of the total of 78 confirmed mutants, 41 demonstrated a reduced susceptibility phenotype and 37 a supersusceptibility (i.e., altered intrinsic resistance) phenotype, with 6 mutants demonstrating a mixed phenotype, depending on the antibiotic. Only three mutants demonstrated reduced (PA0908) or increased (*glnK* and *ftsK*) susceptibility to all three antibiotics. Overall, the mutant profiles of susceptibility suggested distinct mechanisms of action and resistance for the three antibiotics despite their similar structures. More detailed analysis indicated important roles for novel and known β-lactamase regulatory genes, for genes with likely involvement in barrier function, and for a range of regulators of alginate biosynthesis.

Pseudomonas aeruginosa is an important opportunistic pathogen and a leading cause of nosocomial infections (32, 41) and is the major cause of morbidity and mortality among individuals affected by cystic fibrosis (CF) (33). Infections caused by this opportunistic pathogen are difficult to eradicate due to its high intrinsic resistance to different classes of antibiotics. Treatment of patients is further complicated by the emergence of multidrug resistance arising principally from mutations, but also through acquisition of plasmids with antibiotic resistance determinants (32, 41).

β-Lactam antibiotics are among the main antibiotics currently used in anti-pseudomonal therapy (18, 56). The killing mechanism of β-lactams is initiated by binding to cell wall transpeptidases (penicillin-binding proteins [PBPs]), thus blocking an important step in peptidoglycan biosynthesis (59). This family of antibiotics includes penicillins, cephalosporins, monobactams, and carbapenems. Resistance to β-lactams commonly results from drug inactivation by β-lactamases, drug extrusion through efflux pumps, changes in outer membrane permeability, and modification of PBPs (46). However, recent publications have demonstrated that a myriad of genetic determinants modulate susceptibility to antibiotics, aside from those responsible for typical antibiotic resistance mechanisms, like the ones described above (8, 16, 19, 52), especially when one considers mutations causing modest changes in the MIC (e.g., 2-fold). Furthermore, β-lactam antibiotics are known to affect global gene expression, suggesting that the response to these drugs entails many different genes (1, 6).

To identify novel genetic determinants involved in susceptibility to β-lactams, we screened a comprehensive *P. aeruginosa* mutant library (31) for changes in the MICs of imipenem and meropenem (carbapenems) and ceftazidime (a cephalosporin). Our findings demonstrated that mutations in a broad array of genes belonging to different functional families can modulate the susceptibility of *P. aeruginosa* to these antibiotics. This study contributes to our understanding of how pathogens respond and become resistant to β-lactam antibiotics, revealing certain mutations that, because they cause modest changes in the MIC, may be missed in examination of clinical strains but likely contribute to the stepwise development of resistance in the clinic.

MATERIALS AND METHODS

Screening for altered susceptibility to imipenem, ceftazidime, and meropenem. A comprehensive *P. aeruginosa* PA14 mutant library (31) was screened for genes involved in intrinsic resistance (reduced MIC upon mutation) or reduced susceptibility (increased MIC upon mutation) to three different β-lactam antibiotics. A total of 5,850 mutants corresponding to 4,596 predicted PA14 genes were screened using the agar dilution method as previously described (8, 16, 52). Briefly, overnight cultures grown in 96-well plates in LB medium at 37°C were diluted and used to inoculate Mueller-Hinton agar plates containing the antibiotic of choice. The MIC for the PA14 wild-type strain under these conditions was 1 μg/ml for imipenem and ceftazidime and 0.25 μg/ml for meropenem. Imipenem and ceftazidime were used at 2-fold increasing concentrations ranging from 0.5 to 16 μg/ml. Meropenem was used at 0.5 and 1 μg/ml. The plates were incubated at 37°C for at least 18 h before the results were read. Mutants exhibiting at least a 2-fold increase or decrease in MIC with respect to the wild type were considered less susceptible or more susceptible to a particular antibiotic and thus revealed mutational and intrinsic resistance, respectively. The screening was repeated two more times with those mutants that showed a change in susceptibility to any of the three antibiotics used.

Determination of antibiotic susceptibility. Imipenem, ceftazidime, and meropenem Etest strips (AB Biodisk, Solna, Sweden) were utilized, according to the manufacturer's recommendations, to verify the results obtained in the screening. Cultures were grown overnight in LB at 37°C and diluted in sterile 0.85% NaCl

* Corresponding author. Mailing address: Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Darwin 3, Cantoblanco, Madrid, Spain, Phone: 34 91 5854542. Fax: 34 92 5854506. E-mail: jlmtnez@cnb.csic.es.

‡ Present address: AiCuris GmbH and Co. KG, Friedrich-Ebert-Str. 475, 42117 Wuppertal, Germany.

† C.A.-O. and I.W. contributed equally to this work.

▽ Published ahead of print on 2 August 2010.

solution to yield an inoculum of approximately 1×10^7 CFU/ml. Mueller-Hinton agar plates were inoculated with 100 μl of this dilution, strips were placed on the surface (one strip per plate), and the plate was then incubated at 37°C for 18 h. Each set of determinations included a plate containing the wild-type strain *P. aeruginosa* PA14 as a control for antibiotic susceptibility, in such a way that the MICs for PA14 were repeated 124 times for ceftazidime, with a mean of 0.78 and a modal value of 0.75; 113 times for imipenem, with a mean of 0.44 and a modal value of 0.5; and 192 times for meropenem, with a mean of 0.13 and a modal value of 0.125. Given the consistency of these results, the MICs were considered the modal values of all determinations. Ceftazidime MIC values were also determined using the broth microdilution method according to CLSI guidelines (62). To detect subtle changes in susceptibility, a narrower, linear range of concentrations of ceftazidime were tested, which included 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 16 μg/ml, for the mutants presenting either changes in ceftazidime susceptibility or increased levels of β-lactamase activity.

Determination of β-lactamase activity. Cells were grown to an optical density at 600 nm (OD_{600}) of 1.0, harvested by centrifugation, and resuspended in 0.1 M KH_2PO_4-Na_2HPO_4 (pH 7.0) buffer. Crude protein extracts were prepared by two freeze-thaw cycles, followed by sonication on ice. The β-lactamase activity was quantified spectrophotometrically by measuring the change in absorbance at 485 nm using the chromogenic β-lactamase substrate nitrocefin at 50 μM (Oxoid, Basingstoke, United Kingdom) and 0.1 M KH_2PO_4-Na_2HPO_4 (pH 7.0) as the test buffer. The protein content of each extract was determined using the bicinchoninic acid (BCA) method with bovine serum albumin as a standard. *P. aeruginosa* mutants from the PA14 library (31) and the mini-Tn*5-luxCDABE* PAO1 mutant library (30) were utilized to confirm the antibiotic susceptibility phenotypes of mutant alleles.

Complementation of *algU*, *mucD*, *dacB*, and *mpl* mutants. The broad-host-range cloning vector pBBR1MCS-3 (27) was utilized to clone *algU* and *mucD*. To complement the *algU* mutant, the *algU-mucABCD* region was amplified by PCR, including 111 bp upstream of the *algU* translational start site to permit transcription from the native promoter (12). For complementation of the *mucD* mutant, the *mucD* gene sequence, including 54 bp upstream containing the translational start site, was amplified. Both sequences were digested with ApaI and SacI and cloned into pBBR1MCS-3. In the case of *mucD*, transcription was enabled by the *lac* promoter in pBBR1MCS-3. The broad-host-range cloning vector pUCP27 (53) was used to clone the *dacB* and *mpl* genes, including 483 and 313 bp, respectively, upstream of the translational start sites.

Data analysis. Venn diagrams were generated using the Venny tool from BioinfoGP (44). Hierarchical clustering (14) was performed using freely available software (http://rana.lbl.gov/EisenSoftware.htm).

RESULTS AND DISCUSSION

Overview of the screening results. Screening of existing individual mutants in the *P. aeruginosa* PA14 mutant library using the agar dilution method allowed us to identify mutants exhibiting changes in MIC as small as 2-fold for each of the antibiotics imipenem, ceftazidime, and meropenem. To ensure the reliability of our results, the screening was performed independently in our two laboratories, and MICs were determined at least three times. Furthermore, the results were verified by an agar diffusion method using the Etest (62), and mutants with changes in MIC that could not be reproduced were discarded. These safeguards, together with the finding of mutations in an operon relationship (e.g., PA5001 to PA5005) and the isolation of mutations in genes for which roles in susceptibility to β-lactams have been described (see below), added credence to our screening. Some of the mutations might reduce the bacterial growth rate. This might be relevant for determining their antibiotic susceptibility for two reasons. Slow-growing bacteria are less susceptible to bactericidal antibiotics (29). On the other hand, the inoculum size is relevant for the activity of beta-lactams (50, 51). To address whether these issues might bias our results, the growth rates of all mutants were measured. Only 10 of the mutants presented a relevant increase in their duplication times (>50%) in comparison with the wild-type strain. Two of these mutants were excluded from further studies after Etest assays. Of the remaining eight mutants, only one (PA2797) was less susceptible than the wild type to one antibiotic (ceftazidime; MIC, 3 μg/ml), indicating that the observed phenotypes are not just the consequence of a change in growth rate. For all selected mutants, the MICs to the three β-lactams were further verified by Etest. To avoid differences in inoculum sizes that might influence the assays for those mutants with changes in their growth rates, equal numbers of cells were seeded on each plate (see Materials and Methods). In a few cases, the Etest allowed the detection of changes in antibiotic susceptibility that were not evident in the initial screening. Since the Etest is a more robust method to detect small differences in MICs than doubling-dilution methodologies and the results were verified in triplicate by Etest, those phenotypes were included in the results (Tables 1 and 2).

Since we wanted to analyze even small changes in susceptibility, we considered those mutants presenting reduced susceptibility to the tested antibiotics, even if the MIC did not reach the breakpoint required for the clinical definition of resistance. This so-called low-level resistance (4) is the hallmark for eventual acquisition of clinically relevant resistance to antibiotics, so that deciphering the mechanisms of low-level resistance is necessary to understand in depth antibiotic resistance in bacterial pathogens (4, 35, 36). We found that mutations in 78 different *P. aeruginosa* PA14 loci resulted in changes in MICs, with respect to the wild-type strain, with at least one out of the three β-lactam antibiotics we utilized in our screening (Tables 1 and 2). Of these, 41 demonstrated a reduced susceptibility phenotype (Table 1) and 37 a supersusceptibility phenotype (Table 2), with 6 mutants demonstrating a mixed phenotype, depending on the antibiotic. To identify common, as well as unique, genetic determinants that may contribute to intrinsic antibiotic resistance or sensitivity, a Boolean analysis of the results was performed and displayed using Venn diagrams (Fig. 1).

In agreement with studies examining the resistomes of other antibiotics (8, 16, 19, 52), the majority of the mutants showed a modest (around 2-fold) change in MIC. Small changes in MIC values are often not taken into consideration. However, it has been demonstrated that the accumulation of mutations with modest individual contributions to the MIC can lead to significant antibiotic resistance (5, 15, 19, 42). Furthermore, these types of mutations are likely the basis for MIC creep, which can be defined as the constant rise over time in the basal intrinsic resistance of an average isolate of a given bacterial species (55).

Overall, our results show that a wide variety of gene products modulate the susceptibility of *P. aeruginosa* to β-lactams. Together with similar results recently published for other antibiotics (8, 16, 19, 52), this supports the concept that intrinsic resistance to antibiotics is the result of the concerted actions of several elements (36). While it is already well established that outer membrane protein mutations, efflux, and β-lactamase collaborate to determine susceptibility or resistance to β-lactams, these studies indicate a substantial array of new elements that also modulate susceptibility.

In spite of their different spectra of activity and their differential sensitivities to various β-lactamases (46), imipenem,

TABLE 1. Mutations that decrease the susceptibility of *P. aeruginosa* to β-lactams

PAO1 ortholog or PA14 no.[a]	Gene name and/or description	MIC (μg/ml)[b,c]		
		CAZ	IPM	MEM
PA14	Wild-type strain	0.75	0.5	0.125
PA0479	Putative transcriptional regulator, LysR family	**2**	**1**	0.047[e]
PA0667	Putative metallopeptidase	**2**[d]	0.5	0.19
PA0807	*ampDh3*; hypothetical protein	0.75	**1**	**0.25**[d]
PA0908	Hypothetical protein	**2**	**1.5**	**0.25**[d]
PA0958	*oprD*; basic amino acids and peptides and imipenem outer membrane porin OprD	0.75	**2**	**1**
PA1348	Conserved hypothetical protein	**1.5**[d]	0.5	**0.5**
PA14_15600	Conserved hypothetical protein	**1.5**	0.5	0.125
PA14_23420	Putative zinc-binding dehydrogenase	**2**	0.38	0.125
PA14_23430	Putative heparinase	**2**	0.38	0.125
PA14_43090	Hypothetical protein	**2**	0.38	0.125
PA14_06490	Hypothetical protein	**2**	0.38	0.125
PA1553	Putative cytochrome *c* oxidase, cbb3 type, subunit II	0.75	**1**	0.125
PA2023	*galU*; UTP-glucose-1-phosphate uridylyltransferase	**3**[d]	**0.094**	**0.38**
PA2487	Hypothetical protein	**2**[d]	0.5	0.125
PA2621	*clpS*; putative cytoplasmic protease	**4**	0.38	0.19
PA2797	Putative anti-anti-sigma factor	**3**	0.5	0.094
PA3141	*wbpM*; nucleotide sugar epimerase/dehydratase WbpM	**2**	0.5	0.125
PA3145	*wbpL*; putative glycosyltransferase L	**2**	0.38	0.094
PA3247	Putative aspartyl aminopeptidase	**2**	0.38	0.125
PA3259	Conserved hypothetical protein	**1.5**	0.5	0.125
PA3520	Putative periplasmic metal-binding protein	**1.5**	0.5	**0.38**[d]
PA3589	Acetyl-coenzyme A (CoA) acetyltransferase (thiolase)	**2**	0.5	0.125
PA3620	*mutS*; DNA mismatch repair protein MutS	**2**	0.38	0.125
PA3667	Putative cysteine sulfinate desulfinase	**3**	**0.75**	0.125
PA3704	*wspE*; putative chemotaxis protein	**2**	0.5	**0.25**[d]
PA3721	*nalC*; putative transciptional regulator	**1.5**[d]	0.5	**0.38**[d]
PA4109	*ampR*; transcriptional regulator AmpR	**4**	**0.25**	0.125
PA4402	*argJ*; *N*-acetylglutamate synthase	**3**	0.38	0.19
PA4459	Conserved hypothetical protein	**1.5**	**0.25**	0.125
PA4527	*pilC*; type 4 fimbrial biogenesis protein	**2**	0.38	0.125
PA4550	*fimU*; type 4 fimbrial biogenesis protein	**2**[d]	0.5	0.125
PA4748	*tpiA*; triosephosphate isomerase	**1.5**[d]	0.38	**0.25**[d]
PA4946	*mutL*; DNA mismatch repair protein MutL	**1.5**	0.38	0.094
PA5000	*wapR*; putative glycosyl transferase	**4**	0.5	**0.38**[d]
PA5001	Conserved hypothetical protein	**3**	0.38	**0.38**[d]
PA5002	Hypothetical protein	**1.5**[d]	0.38	0.094
PA5003	Conserved hypothetical protein	**3**	0.5	**0.25**[d]
PA5005	Putative carbamoyltransferase	**3**	0.25	0.125
PA5038	*aroB*;3-dehydroquinate synthase	**3**	0.38	**0.25**
PA5192	*pckA;* phosphoenolpyruvate carboxykinase	0.5	0.38	**0.25**
PA5443	*uvrD*; DNA helicase II	**1.5**	0.5	0.094

[a] Mutant in PAO1 ortholog. Some mutaions were in PA14 genes that have no PAO1 ortholog.
[b] MIC determined by Etest. Changes in MICs of ≥2-fold are in boldface.
[c] CAZ, ceftazidime; IPM, imipenem; MEM, meropenem.
[d] A change in MIC was observed only with the Etest.
[e] The screening revealed a decrease in susceptibility rather than an increase.

ceftazidime, and meropenem all affect cell wall synthesis by interacting with specific penicillin-binding proteins and murein hydrolases (59); therefore, we expected to identify a core set of genetic determinants involved in susceptibility to this family of antibiotics. However, the Boolean analysis of our results and hierarchical clustering of the phenotypes (Fig. 1) indicated that the overlap among the different phenotypes is minimal. Indeed, we found only one mutant with reduced susceptibility to all three antibiotics (PA0908; MICs, 2, 1.5, and 0.25 μg/ml for ceftazidime, imipenem, and meropenem, respectively) and two mutants with increased sensitivity to all three antibiotics (*glnK*, with MICs of 0.5, 0.25, and 0.047 μg/ml for ceftazidime, imipenem, and meropenem, respectively, and *ftsK*, with MICs of 0.25, 0.19, and 0.064 μg/ml for ceftazidime, imipenem, and meropenem, respectively) (Tables 1 and 2). A further 14 mutants showed reduced susceptibility to two antibiotics, and 12 showed increased susceptibility to two antibiotics (Fig. 1). Despite the structural similarities between meropenem and imipenem, there was a larger number of mutants showing decreased susceptibility to ceftazidime and meropenem than mutants with reduced susceptibility to both imipenem and meropenem (Table 1).

Mutants with reduced susceptibility and altered uptake or efflux. In our screen, there was a larger number of mutants resulting in a decrease in susceptibility to ceftazidime than to the other two antibiotics (Table 1). This suggests that *P. aeruginosa* might be more likely to develop mutation-driven resistance to ceftazidime than to imipenem or meropenem (34). This is important from a clinical perspective, since all three antibiotics are used in antipseudomonal therapy (18). For

TABLE 2. Mutations that increase the susceptibility of *P. aeruginosa* to β-lactams

PAO1 ortholog or PA14 no.[a]	Gene name and/or description	MIC (μg/ml)[b,c]		
		CAZ	IPM	MEM
PA14	Wild-type strain	0.75	0.5	0.125
PA0011	Putative 2-OH-lauroyltransferase	**0.38**[d]	0.38	0.125
PA0401	Noncatalytic dihydroorotase-like protein	**0.38**	0.38	**0.064**
PA0402	*pyrB*; aspartate carbamoyltransferase	**0.38**	0.38	0.125
PA0420	*bioA*, adenosylmethionine-8-amino-7-oxononanoate aminotransferase	**0.5**	**0.125**	0.094
PA0427	*oprM*; major intrinsic multiple antibiotic resistance efflux outer membrane protein	**0.38**	0.38	**0.023**
PA0503	Putative biotin synthesis protein BioC	**0.5**[d]	**0.19**	0.125
PA0764	*mucB*; negative regulator for alginate biosynthesis	0.75	**0.25**	0.125
PA0766	*mucD*; serine protease MucD precursor	**0.38**	**0.25**	0.125
PA0770	*rnc*; RNase III	**0.5**	**0.75**[d]	**0.047**
PA1011	Putative lipoprotein	**0.5**	0.5	0.19
PA1195	Putative dimethylarginine dimethylaminohydrolase	**0.5**	0.38	0.125
PA14_07490/07510	Intergenic region	**0.5**	0.5	0.094
PA14_59780	*rcsC*; two- component system kinase sensor	**0.38**	0.5	0.125
PA1483	*cycH*; cytochrome *c*-type biogenesis protein	**0.5**	0.5	0.125
PA2128	*cupA1*; fimbrial subunit CupA1	**0.5**	0.38	0.125
PA2615	*ftsK*; cell division/stress response protein	**0.25**	**0.19**	**0.064**
PA2963	Putative aminodeoxychorismate lyase	0.75	**0.25**	0.125
PA2970	*rpmF*; 50S ribosomal protein L32	**0.5**	0.5	0.125
PA3050	*pyrD*; dihydroorotate dehydrogenase	**0.5**	**0.25**	0.094
PA3262	*fklB*; put. peptidyl-prolyl *cis-trans*-isomerase, FkbP type	0.75	**0.25**	0.125
PA3433	Putative transcriptional regulator, LysR family	**0.5**	**0.19**	0.125
PA3649	Putative membrane-associated zinc metalloprotease	**0.5**	**0.25**	0.125
PA3800	Conserved hypothetical protein	**0.25**	0.38	0.125
PA3818	*suhB*; extragenic suppressor protein SuhB	**0.5**	0.38	**0.064**
PA3978	Hypothetical protein	0.75	**0.094**	0.125
PA4005	Conserved hypothetical protein	0.75	**0.064**	0.094
PA4007	*proA*; probable γ-glutamyl phosphate reductase	0.75	**0.19**	0.125
PA4069	Putative dTDP-4-rhamnose reductase-related protein	0.75	**0.25**	**0.064**
PA4088	Putative aminotransferase	**0.25**	0.38	0.125
PA4269	*rpoC*; DNA-directed RNA polymerase b chain	**0.5**	0.5	0.094
PA4393	Putative permease	0.75	**0.25**	0.19
PA4745	*nusA*; N utilization substance protein A	0.75	**0.25**	0.125
PA4753	Putative RNA-binding protein	**0.25**	0.5	**0.064**
PA5130	Putative rhodanese-like domain protein	0.75	**0.19**	0.125
PA5174	Putative β-ketoacyl synthase	1	**0.125**	0.125
PA5288	*glnK*; nitrogen regulatory protein PII-2	**0.5**[d]	**0.25**	**0.047**
PA5366	*pstB*; phosphate ABC transporter, ATP binding	0.75	**0.25**[d]	0.125

[a] Mutant in PAO1 ortholog. Some mutaions were in PA14 genes that have no PAO1 ortholog.
[b] MIC determined by Etest. Changes in MICs of ≥2-fold are in boldface.
[c] CAZ, ceftazidime; IPM, imipenem; MEM, meropenem.
[d] A change in MIC was observed only with the Etest.

many of the mutants that exhibited a decrease in susceptibility to ceftazidime alone, the mutations were in genes involved in cell wall and lipopolysaccharide (LPS) biosynthesis, as well as in genes encoding membrane proteins (*wapR* and PA5001 to PA5005) (Table 1), suggesting a decrease in antibiotic penetration as the basis for the observed increase in the MIC.

As anticipated, well-defined mutants influencing antibiotic uptake were also isolated, a result that further attests to the reliability of our screening. Nonhydrolytic resistance to third-generation cephalosporins like ceftazidime often results from antibiotic extrusion through efflux pumps, such as MexAB-OprM (39, 46). Accordingly, we found that a mutation in the gene coding for the NalC negative regulator (9) of the major intrinsic multidrug efflux pump, MexAB-OprM, made *P. aeruginosa* less susceptible (Table 1) while an *oprM* mutant was more susceptible to ceftazidime (Table 2) (MICs, 1.5 and 0.38 μg/ml, respectively). Imipenem and meropenem differ in a number of structural features and in their antipseudomonal activities, with meropenem being the more efficient of the two carbapenems (10, 18). Nevertheless, the OprD outer membrane porin serves as the major route of entry for both imipenem and meropenem, and resistance in clinical isolates is usually associated with lower expression or loss of this porin (45). As expected, an *oprD* mutant identified through our screening exhibited a decrease in susceptibility to both carbapenems (the MIC of imipenem is 2 μg/ml, and that of meropenem is 1 μg/ml) (Table 1).

Mutants with reduced susceptibility and altered LPS compositions. A mutation in PA5000, which encodes the WapR α-1,3-rhamnosyltransferase involved in the biosynthesis of the LPS core oligosaccharide (47), made *P. aeruginosa* less susceptible to ceftazidime and meropenem (the MIC of ceftazidime is 4 μg/ml, and that of meropenem is 0.38 μg/ml) (Table 1). It was previously reported that a *wapR* mutant lacked α-1,3-linked L-rhamnose and therefore produced LPS with no O antigen (47). Interestingly, genes PA5001, PA5002, PA5003, and PA5005, which are part of a large LPS core oligosaccharide gene cluster, were also detected in the screen for reduced

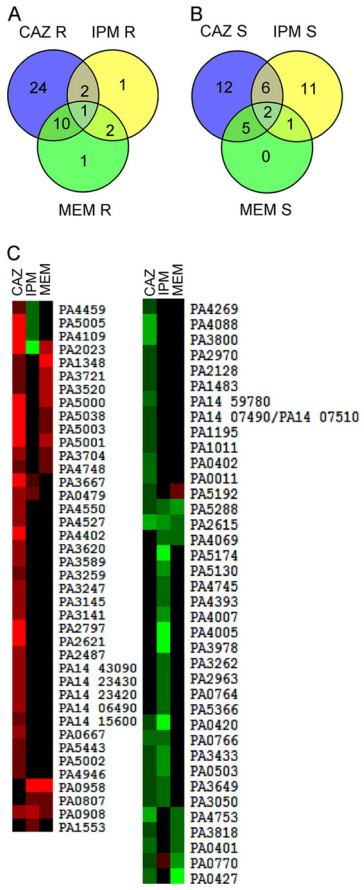

FIG. 1. Global analysis of the mutants that exhibited a decrease or an increase in susceptibility to at least one β-lactam. Abbreviations for antibiotics are as follows: IPM, imipenem; CAZ, ceftazidime; and MEM, meropenem. (A and B) Venn diagrams resulting from a Boolean analysis of the phenotypes of the mutants. (A) Venn diagram created with mutants showing a decrease in susceptibility to at least one β-lactam. R stands for "resistant" mutants. (B) Venn diagram created with mutants showing an increase in susceptibility to at least one β-lactam. S stands for "sensitive" mutants. (C) Analysis of the association of the phenotypes of the different mutants. The analysis was performed by hierarchical clustering of the MIC fold changes shown in Tables 1 and 2. The green bars stand for mutants with an increase in susceptibility; the red bars stand for mutants with a decrease in susceptibility. The heat map was constructed by using Cluster and TreeView software (14).

susceptibility to ceftazidime, and some mutants were cross resistant to meropenem (25) (Table 1). The increase in the MIC (3 μg/ml with ceftazidime and 0.38 μg/ml with meropenem), due to an insertion in PA5001, a putative glycosyltransferase gene, observed with the Etest was also confirmed in broth dilution assays (a 3-fold increase in the ceftazidime MIC versus the PA14 wild type). The respective PAO1 mutant from the mini-Tn*5-luxCDABE* mutant library (27) also showed a 2-fold increase in MIC versus the PAO1 wild type. Other genes involved in LPS biosynthesis that led to increased ceftazidime MIC values were *wpmM* (PA3141; 2 μg/ml), *wbpL* (PA3145; 2 μg/ml), *wspE* (PA3704; 2 μg/ml), and *galU* (PA2023; 3 μg/ml) (Table 1). Alterations in LPS composition might affect outer membrane barrier properties and hence antibiotic penetration, thus reducing their efficacy (17, 21).

Mutations in genes that affect β-lactamase production. We were particularly interested in identifying novel genes that are involved in the regulation of the *Pseudomonas* chromosomally encoded, inducible AmpC β-lactamase. Thus, mutants that showed an increased ceftazidime MIC on agar and in microdilution MIC assays, with the exception of mutants with a previously described mutator phenotype (63), were tested for β-lactamase overexpression. Table 3 shows the mutants with 2-fold or higher β-lactamase activity compared to the wild-type strain. Four of the PA14 high-level β-lactamase-producing mutants (PA0090, PA2023, PA2989, and PA3589) were found only in the PA14 background but could not be confirmed with mutants in the PAO1 background (data not shown). β-Lactamase overproduction in the respective PA14 mutants might be due to an accumulation of secondary mutations in these strains, and consistent with this interpretation, the PA2023 (*galU*) and PA3589 mutants had elevated mutation frequencies (data not shown). Alternatively, this may result from differences in the insertion point of the transposon or as a consequence of the different genetic backgrounds of the PAO1 and PA14 strains. The fact that intrinsic resistance has some degree of strain specificity is supported by other studies (11, 13) and by the findings from our screen of elements that contributed to the β-lactam resistome of *P. aeruginosa* PA14 but were absent in the genome of strain PAO1 (Tables 1 and 2). Four transposon mutants, *ampR*, *ampD*, *dacB*, and *mpl*, were found to lead to β-lactamase overproduction in both *P. aeruginosa* strains.

TABLE 3. *P. aeruginosa* mutants with reduced ceftazidime susceptibility and increased β-lactamase activity

Deletion mutant PA no.	Gene	Gene description	Specific β-lactamase activity[a]		Fold increase in β-lactamase activity[b]	
			PA14	PAO1	PA14	PA01
PA14 wild type			4.9		1	
PAO1 wild type				6.9		1
PA3047	*dacB*	D-Alanyl-D-alanine carboxypeptidase, PBP4	110	274	22	40
PA3047 (pUCP27-*dacB*$^+$)		Complemented *dacB* mutant	3.8		0.7	
PA4020	*mpl*	UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	96	168	19.5	24
PA4020 (pUCP27-*mpl*$^+$)		Complemented *mpl* mutant	7.3		1.5	
PA4109	*ampR*	Transcriptional regulator AmpR	257	111	52	16
PA4522	*ampD*	*N*-Acetyl-anhydromuranmyl-L-alanine amidase	31	74	6.4	11

[a] Expressed as nmol nitrocefin hydrolyzed/min · mg protein.
[b] Fold change of the mutant in comparison with its wild-type parental strain.

AmpR has been identified in all bacteria with inducible AmpC β-lactamase expression, including *P. aeruginosa*. Anhydromuropeptides that accumulate on induction with β-lactam antibiotics or during perturbation of the peptidoglycan-recycling pathway (*ampD* mutations) turn AmpR into an activator of transcription (2, 61). It has been shown that both the ceftazidime-resistant PA14 *ampR* and the PAO1 *ampR* mutants produced significantly higher (16 to 52 times) levels of β-lactamase (26) (Table 3). The increased β-lactamase activity of these mutants has been attributed to increased production of the β-lactamases AmpC (40) and PoxB (26). Since production of high levels of PoxB does not change ceftazidime susceptibility (20), we conclude that the increased ceftazidime MIC in the *ampR* mutants (3-fold in PA14) (Table 3) might be explained by increased production of AmpC β-lactamase. Besides reduced susceptibility to ceftazidime, the strain PA14 *ampR* mutant showed an increase in imipenem susceptibility (Table 1). Data from Moya et al. for strain PAO1 are in agreement with this finding (40). Since we found that the PA14 *poxB* mutant had no alteration in susceptibility to either imipenem or meropenem (data not shown), the observed increase in susceptibility to carbapenems might be due to other genes regulated by AmpR, a hypothesis that is supported by the role of AmpR as a global regulator in *P. aeruginosa* (26).

As expected (24), strain PA14 and PAO1 *ampD* mutants overproduced the AmpC β-lactamase (6.4-fold and 11-fold, respectively). *P. aeruginosa* encodes three AmpD homologs in its genome that contribute to AmpC induction, likely by increasing the amounts of the inducing ligand for AmpR (24). In our screen, the *ampDh3* mutant was determined to be less susceptible to imipenem (MIC, 1 μg/ml) (Table 1) and meropenem (MIC, 0.25 μg/ml) (Table 1) than the wild-type strain, again pointing to the fact that AmpR might be involved in regulating carbapenem susceptibility genes. Although the hydrolysis of imipenem and meropenem by *P. aeruginosa* AmpC is below the limits of detection (48), and it has been stated that AmpC alone does not account for the increased susceptibility to carbapenems observed in a mutant that overproduces the MDR efflux pump *mexCD-oprJ* (64), it has also been shown that this β-lactamase can contribute to intrinsic resistance by its interplay with other mechanisms, such as the permeability of the outer membrane and the activity of the MDR efflux pumps (38, 43). Thus, the possibility that AmpC, in cooperation with other elements, might play a role in the observed reduced susceptibility to carbapenems of this mutant cannot be ruled out.

A recent study showed that deletion of the nonessential penicillin-binding protein PBP4, a D-alanyl-D-alanine carboxypeptidase encoded by PA3047 (*dacB*), triggers overexpression of *ampC* (40). In agreement with this report, we found that a *dacB* mutant was less susceptible to ceftazidime, with a MIC of 6 μg/ml versus the 2 μg/ml of wild-type PA14 (determined by broth microdilution using a linear range of concentrations; see Materials and Methods), and led to 22- to 40-fold-increased β-lactamase activity (i.e., greater than an *ampD* mutant). Furthermore, we found that this change in the levels of β-lactamase activity could be complemented with the cloned *dacB* gene (Table 3). Consistent with this complementation, the MIC of ceftazidime was restored to the levels of the wild-type strain, 2 μg/ml, upon complementation. Since it was not known whether other nonessential PBPs also contributed to AmpC overproduction in *P. aeruginosa*, we determined the β-lactamase activities of the PA14 mutants *pbpG* (PA0869), encoding a D-alanyl-D-alanine endopeptidase, and *dacC* (PA3999), a second D-ala-D-ala-carboxypeptidase. Both mutants showed β-lactamase activities comparable to that of the wild-type strain (data not shown).

Disruption of the gene PA4020 (*mpl*) caused a small increase in the ceftazidime MIC (3 μg/ml) in comparison with the wild-type strain (2 μg/ml) and an approximately 20-fold increase in β-lactamase activity in both PA14 and PAO1. Susceptibility to ceftazidime and β-lactamase activity were restored to the levels of the wild-type strain by reintroducing the *mpl* gene on a multicopy plasmid. Mpl is a UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase, an enzyme that is active in the peptidogylcan-recycling pathway. In an *Escherichia coli mpl* mutant, a 1.7-fold decrease in the level of the UDP-MurNAc-pentapeptide pool was observed compared to the wild type (23). In *Citrobacter freundii*, *in vitro* transcription assays demonstrated that this murein precursor acts as a repressing ligand for AmpR (57). It has been suggested that a similar situation might occur in *P. aeruginosa* (32, 40). However, no experimental evidence had been provided. The substantial overproduction of the AmpC β-lactamase in the *P. aeruginosa mpl* and *ampR* mutants, as seen in this study, supports these suggestions (32, 40), indicating that *P. aeruginosa* AmpR might be regulated through the peptidoglycan-recycling pathway, as in the case of *C. freundii* (57).

Mutants showing increased susceptibility to β-lactams. One of the two mutants showing an increase in susceptibility to all three antibiotics was the *ftsK* mutant (MICs, 0.25, 0.19, and 0.064 μg/ml for ceftazidime, imipenem, and meropenem, respectively) (Table 2). FtsK is a multidomain protein involved in cell division and possibly in the final stages of peptidoglycan biosynthesis (60). The hypersusceptibility phenotype is not exclusive to β-lactams, as a *P. aeruginosa ftsK* mutant has been shown to be more susceptible to other families of antibiotics (8, 13). PA0011 encodes a putative 2-OH-lauroyltransferase involved in lipid A biosynthesis and is a homolog of *E. coli* LpxL. Susceptibility to ceftazidime (MIC, 0.38 μg/ml) (Table 2), therefore, might be due to an increase in the cell permeability, since an increase in antibiotic susceptibility has also been reported in an *E. coli lpxL* mutant (58).

The number of mutants more susceptible to ceftazidime was comparable to the numbers obtained with imipenem (Fig. 1). The former included a mutant in *rcsC* (MIC, 0.38 μg/ml) (Table 2), which encodes a sensor kinase that forms part of the Rcs phosphorelay found in a number of pathogenic bacteria (22). In *E. coli*, the Rcs pathway responds to damage to the peptidoglycan layer and contributes to intrinsic resistance to β-lactams (19, 28). According to our data, this system might have a similar function in *P. aeruginosa.*

Mutations in genes involved in alginate production. *P. aeruginosa* CF isolates often bear mutations leading to overproduction of alginate exopolysaccharide that result in a highly mucoid phenotype that is proposed to contribute to the viscous mucus in the lungs of individuals with CF (33). The expression of genes required for alginate production is under the control of the alternative sigma factor AlgU (49). MucA and MucB negatively regulate AlgU by sequestering it until the appropri-

TABLE 4. Imipenem MICs for *algU*- and *mucD*-complemented strains

Strain	MIC[a] (μg/ml)
Wild type	0.5
Wild type(pBBR1 MCS-3)	0.5
algU	0.38
algU(pBBR1 MCS-3)	0.38
algU(pBBR-*algU*)	0.5
mucD	0.25
mucD(pBBR1 MCS-3)	0.25
mucD(pBBR-*mucD*)	0.5

[a] MIC determined by Etest in triplicate. The Mueller-Hinton plates contained tetracycline at 20 μg/ml for plasmid maintenance.

ate environmental signals cue alginate biosynthesis (49). In addition, AlgU is negatively regulated by the periplasmic serine proteases MucD and AlgW (7). Predictably, *mucA*, *mucB*, *mucD*, and *algW* mutants overproduce alginate when grown under the appropriate conditions (7, 49). In our screen, mutants in *algU*, *mucB*, *mucD*, and *algW* were found to be more susceptible to imipenem. The phenotypes of *mucB* and *mucD* mutants were confirmed by Etest assays (MIC for both, 0.25 μg/ml) (Table 2), whereas *algU* and *algW* mutants presented a consistent reduction in their MIC values that was below the threshold used in our study. As *mucA* and *mucC* mutants are not available in the PA14 mutant library, we did not screen their phenotype. Confirming the involvement of these determinants in the susceptibility to imipenem of *P. aeruginosa*, expression of cloned *algU* (from its native promoter) and *mucD* restored the imipenem MICs for the corresponding mutants to that of the wild type (0.5 μg/ml) (Table 4). It is known that cell wall-inhibitory antibiotics induce alginate biosynthesis through a mechanism that requires AlgU (1, 65). The small, yet consistent, increase in susceptibility to imipenem exhibited by the *algU* mutant is in agreement with these studies. The results obtained with the *mucB* and *mucD* mutants suggest that this is a complex signaling mechanism that requires the release of AlgU at the appropriate time upon signal recognition. The constant availability of AlgU might interfere with the ability of cells to respond to cell wall damage through altered expression of genes. Interestingly, mucoid clinical isolates have been shown to be more susceptible to some antibiotics (3), and in agreement with our data, a recent study reported the hypersusceptibility of a *mucB* mutant to a variety of antibiotics (13).

Conclusions. Bacteria presenting low-level susceptibility to antibiotics (intrinsic resistance) are a relevant clinical problem. Understanding the mechanisms involved in such resistance, as well as the potential to achieve mutation-driven reduced susceptibility to antibiotics, is a relevant task to fight infections by these microorganisms and to predict the possibility of emergence of resistance (35). This might be particularly relevant for organisms like *P. aeruginosa* that produce chronic infections during which resistance is acquired through mutations (37, 54). Because of this, a number of studies have been recently published on the resistome of *P. aeruginosa* to different families of antibiotics (8, 16, 19, 52). Here, we have presented data on the intrinsic and mutational resistome of this bacterial species to β-lactams. While we found mutants that displayed a decrease or an increase in susceptibility to at least two β-lactams, our results suggest that each antibiotic used in this study has a distinct effect on *P. aeruginosa* and that many determinants modulate the susceptibility to these antibiotics in this opportunistic pathogen. They include a number of mutants with mutations in genes involved in cell wall and LPS biosynthesis, mutants with mutations in genes coding for transporters or efflux determinants, and mutants with altered expression of chromosomally encoded β-lactamases. Interestingly, some of these mutants were also less susceptible to tobramycin (52), indicating that intrinsic resistance to antibiotics lacks specificity to some degree. This is particularly important for CF patients, since current treatment of advanced lung disease often comprises combination therapy of tobramycin associated with a β-lactam (56), and mutations causing coresistance to both antibiotics might be relevant in this situation. The increase in susceptibility observed with the *muc* mutants is also interesting, given that chronic infections caused by mucoid *P. aeruginosa* are characterized by resistance to antibiotic treatment (33).

Together with previously published studies of other antibiotics (8, 16, 19, 52), our results for the β-lactam resistome indicate that the intrinsic and mutational resistomes of *P. aeruginosa* involve a wide array of elements. The fact that mutations in dozens of genes reduced susceptibility to β-lactam antibiotics also indicates that this bacterial species has a high potential to evolve toward resistance.

ACKNOWLEDGMENTS

We thank Jesús Blázquez for sharing the pBBR1MCS-3 cloning vector with us.

Research in J.L.M.'s laboratory was supported by BIO2008-00090 from the Spanish Ministerio de Ciencia e Innovación and KBBE-227258 (BIOHYPO) and HEALTH-F3-2010-241476 (PAR) from the European Union. R.E.W.H. was supported by the Canadian Cystic Fibrosis Foundation. C.A.-O. is the recipient of a JAE contract from CSIC. J.O. is the recipient of a fellowship from Programa Beca Chile, CONICYT. R.E.W.H. holds a Canada Research Chair. I.W. thanks the Juergen Manchot Foundation and the Mukoviszidose e.V., Bonn, Germany (German Cystic Fibrosis Association), for financial support.

REFERENCES

1. **Bagge, N., M. Schuster, M. Hentzer, O. Ciofu, M. Givskov, E. P. Greenberg, and N. Hoiby.** 2004. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. Antimicrob. Agents Chemother. **48:**1175–1187.
2. **Balcewich, M. D., T. M. Reeve, E. A. Orlikow, L. J. Donald, D. J. Vocadlo, and B. L. Mark.** 2010. Crystal structure of the AmpR effector binding domain provides insight into the molecular regulation of inducible AmpC beta-lactamase. J. Mol. Biol. **400:**998–1010.
3. **Balke, B., L. Hoy, H. Weissbrodt, and S. Haussler.** 2004. Comparison of the Micronaut Merlin automated broth microtiter system with the standard agar dilution method for antimicrobial susceptibility testing of mucoid and nonmucoid *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Eur. J. Clin. Microbiol. Infect. Dis. **23:**765–771.
4. **Baquero, F.** 2001. Low-level antibacterial resistance: a gateway to clinical resistance. Drug Resist. Updat. **4:**93–105.
5. **Baquero, F., M. C. Negri, M. I. Morosini, and J. Blazquez.** 1998. Selection of very small differences in bacterial evolution. Int. Microbiol. **1:**295–300.
6. **Blázquez, J., J. M. Gomez-Gomez, A. Oliver, C. Juan, V. Kapur, and S. Martin.** 2006. PBP3 inhibition elicits adaptive responses in *Pseudomonas aeruginosa*. Mol. Microbiol. **62:**84–99.
7. **Boucher, J. C., J. Martinez-Salazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic.** 1996. Two distinct *loci* affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. J. Bacteriol. **178:**511–523.
8. **Breidenstein, E. B., B. K. Khaira, I. Wiegand, J. Overhage, and R. E. Hancock.** 2008. Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. Antimicrob. Agents Chemother. **52:**4486–4491.

9. **Cao, L., R. Srikumar, and K. Poole.** 2004. MexAB-OprM hyperexpression in NalC-type multidrug-resistant *Pseudomonas aeruginosa*: identification and characterization of the *nalC* gene encoding a repressor of PA3720-PA3719. Mol. Microbiol. **53:**1423–1436.
10. **Craig, W. A.** 1997. The pharmacology of meropenem, a new carbapenem antibiotic. Clin. Infect. Dis. **24**(Suppl. 2)**:**S266–S275.
11. **De Groote, V. N., N. Verstraeten, M. Fauvart, C. I. Kint, A. M. Verbeeck, S. Beullens, P. Cornelis, and J. Michiels.** 2009. Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. FEMS Microbiol. Lett. **297:**73–79.
12. **DeVries, C. A., and D. E. Ohman.** 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. J. Bacteriol. **176:**6677–6687.
13. **Dötsch, A., T. Becker, C. Pommerenke, Z. Magnowska, L. Jansch, and S. Haussler.** 2009. Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **53:**2522–2531.
14. **Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein.** 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U. S. A. **95:**14863–14868.
15. **El'Garch, F., K. Jeannot, D. Hocquet, C. Llanes-Barakat, and P. Plesiat.** 2007. Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. **51:**1016–1021.
16. **Fajardo, A., N. Martinez-Martin, M. Mercadillo, J. C. Galan, B. Ghysels, S. Matthijs, P. Cornelis, L. Wiehlmann, B. Tummler, F. Baquero, and J. L. Martinez.** 2008. The neglected intrinsic resistome of bacterial pathogens. PLoS One **3:**e1619.
17. **Ghosh, A. S., A. K. Kar, and M. Kundu.** 1999. Impaired imipenem uptake associated with alterations in outer membrane proteins and lipopolysaccharides in imipenem-resistant *Shigella dysenteriae*. J. Antimicrob. Chemother. **43:**195–201.
18. **Giamarellou, H., and K. Kanellakopoulou.** 2008. Current therapies for *Pseudomonas aeruginosa*. Crit. Care Clin. **24:**261–278.
19. **Girgis, H. S., A. K. Hottes, and S. Tavazoie.** 2009. Genetic architecture of intrinsic antibiotic susceptibility. PLoS One **4:**e5629.
20. **Girlich, D., T. Naas, and P. Nordmann.** 2004. Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **48:**2043–2048.
21. **Godfrey, A. J., L. Hatlelid, and L. E. Bryan.** 1984. Correlation between lipopolysaccharide structure and permeability resistance in beta-lactam-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26:**181–186.
22. **He, J., R. L. Baldini, E. Deziel, M. Saucier, Q. Zhang, N. T. Liberati, D. Lee, J. Urbach, H. M. Goodman, and L. G. Rahme.** 2004. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc. Natl. Acad. Sci. U. S. A. **101:**2530–2535.
23. **Hervé, M., A. Boniface, S. Gobec, D. Blanot, and D. Mengin-Lecreulx.** 2007. Biochemical characterization and physiological properties of *Escherichia coli* UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase. J. Bacteriol. **189:**3987–3995.
24. **Juan, C., B. Moya, J. L. Perez, and A. Oliver.** 2006. Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level beta-lactam resistance involves three AmpD homologues. Antimicrob. Agents Chemother. **50:**1780–1787.
25. **King, J. D., D. Kocincova, E. L. Westman, and J. S. Lam.** 2009. Review: Lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. Innate Immun. **15:**261–312.
26. **Kong, K. F., S. R. Jayawardena, S. D. Indulkar, A. Del Puerto, C. L. Koh, N. Hoiby, and K. Mathee.** 2005. *Pseudomonas aeruginosa* AmpR is a global transcriptional factor that regulates expression of AmpC and PoxB beta-lactamases, proteases, quorum sensing, and other virulence factors. Antimicrob. Agents Chemother. **49:**4567–4575.
27. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
28. **Laubacher, M. E., and S. E. Ades.** 2008. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J. Bacteriol. **190:**2065–2074.
29. **Lee, S. W., E. J. Foley, and J. A. Epstein.** 1944. Mode of action of penicillin: I. Bacterial growth and penicillin activity-*Staphylococcus aureus* FDA. J. Bacteriol. **48:**393–399.
30. **Lewenza, S., R. K. Falsafi, G. Winsor, W. J. Gooderham, J. B. McPhee, F. S. Brinkman, and R. E. Hancock.** 2005. Construction of a mini-Tn*5-luxCDABE* mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes. Genome Res. **15:**583–589.
31. **Liberati, N. T., J. M. Urbach, S. Miyata, D. G. Lee, E. Drenkard, G. Wu, J. Villanueva, T. Wei, and F. M. Ausubel.** 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. U. S. A. **103:**2833–2838.
32. **Lister, P. D., D. J. Wolter, and N. D. Hanson.** 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin. Microbiol. Rev. **22:**582–610.
33. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194–222.
34. **Martinez, J. L., and F. Baquero.** 2000. Mutation frequencies and antibiotic resistance. Antimicrob. Agents Chemother. **44:**1771–1777.
35. **Martínez, J. L., F. Baquero, and D. I. Andersson.** 2007. Predicting antibiotic resistance. Nat. Rev. Microbiol. **5:**958–965.
36. **Martinez, J. L., A. Fajardo, L. Garmendia, A. Hernandez, J. F. Linares, L. Martinez-Solano, and M. B. Sanchez.** 2009. A global view of antibiotic resistance. FEMS Microbiol. Rev. **33:**44–65.
37. **Martínez-Solano, L., M. D. Macia, A. Fajardo, A. Oliver, and J. L. Martinez.** 2008. Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. Clin. Infect. Dis. **47:**1526–1533.
38. **Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, and T. Nishino.** 2001. Hypersusceptibility of the Pseudomonas aeruginosa nfxB mutant to beta-lactams due to reduced expression of the ampC beta-lactamase. Antimicrob. Agents Chemother. **45:**1284–1286.
39. **Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, and T. Nishino.** 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**3322–3327.
40. **Moya, B., A. Dotsch, C. Juan, J. Blazquez, L. Zamorano, S. Haussler, and A. Oliver.** 2009. Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. PLoS Pathog. **5:**e1000353.
41. **Navon-Venezia, S., R. Ben-Ami, and Y. Carmeli.** 2005. Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. Curr. Opin. Infect. Dis. **18:**306–313.
42. **Negri, M. C., M. Lipsitch, J. Blazquez, B. R. Levin, and F. Baquero.** 2000. Concentration-dependent selection of small phenotypic differences in TEM beta-lactamase-mediated antibiotic resistance. Antimicrob. Agents Chemother. **44:**2485–2491.
43. **Okamoto, K., N. Gotoh, and T. Nishino.** 2001. *Pseudomonas aeruginosa* reveals high intrinsic resistance to penem antibiotics: penem resistance mechanisms and their interplay. Antimicrob. Agents Chemother. **45:**1964–1971.
44. **Oliveros, J. C.** 2007 posting date. VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.
45. **Pai, H., J. Kim, J. Kim, J. H. Lee, K. W. Choe, and N. Gotoh.** 2001. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. **45:**480–484.
46. **Poole, K.** 2004. Resistance to beta-lactam antibiotics. Cell Mol. Life Sci. **61:**2200–2223.
47. **Poon, K. K., E. L. Westman, E. Vinogradov, S. Jin, and J. S. Lam.** 2008. Functional characterization of MigA and WapR: putative rhamnosyltransferases involved in outer core oligosaccharide biosynthesis of *Pseudomonas aeruginosa*. J. Bacteriol. **190:**1857–1865.
48. **Queenan, A. M., W. Shang, R. Flamm, and K. Bush.** 2010. Hydrolysis and inhibition profiles of beta-lactamases from molecular classes A to D with doripenem, imipenem, and meropenem. Antimicrob. Agents Chemother. **54:**565–569.
49. **Ramsey, D. M., and D. J. Wozniak.** 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. Mol. Microbiol. **56:**309–322.
50. **Reguera, J. A., F. Baquero, J. C. Perez-Diaz, and J. L. Martinez.** 1991. Factors determining resistance to beta-lactam combined with beta-lactamase inhibitors in *Escherichia coli*. J. Antimicrob. Chemother. **27:**569–575.
51. **Reguera, J. A., F. Baquero, J. C. Perez-Diaz, and J. L. Martinez.** 1988. Synergistic effect of dosage and bacterial inoculum in TEM-1 mediated antibiotic resistance. Eur. J. Clin. Microbiol. Infect. Dis. **7:**778–779.
52. **Schurek, K. N., A. K. Marr, P. K. Taylor, I. Wiegand, L. Semenec, B. K. Khaira, and R. E. Hancock.** 2008. Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **52:**4213–4219.
53. **Schweizer, H. P.** 1994. A method for construction of bacterial hosts for lac-based cloning and expression vectors: alpha-complementation and regulated expression. Biotechniques **17:**452–454, 456.
54. **Smith, E. E., D. G. Buckley, Z. Wu, C. Saenphimmachak, L. R. Hoffman, D. A. D'Argenio, S. I. Miller, B. W. Ramsey, D. P. Speert, S. M. Moskowitz, J. L. Burns, R. Kaul, and M. V. Olson.** 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc. Natl. Acad. Sci. U. S. A. **103:**8487–8492.
55. **Steinkraus, G., R. White, and L. Friedrich.** 2007. Vancomycin MIC creep in non-vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-susceptible clinical methicillin-resistant *S. aureus* (MRSA) blood isolates from 2001–05. J. Antimicrob. Chemother. **60:**788–794.
56. **Taccetti, G., S. Campana, A. S. Neri, V. Boni, and F. Festini.** 2008. Antibiotic therapy against *Pseudomonas aeruginosa* in cystic fibrosis. J. Chemother. **20:**166–169.
57. **Uehara, T., and J. T. Park.** 2002. Role of the murein precursor UDP-N-acetyl-

muramyl-L-Ala-gamma-D-Glu-meso-diaminopimelicacid-D-Ala-D-Ala in repression of beta-lactamase induction in cell division mutants. J. Bacteriol. **184:** 4233–4239.
58. **Vorachek-Warren, M. K., S. Ramirez, R. J. Cotter, and C. R. Raetz.** 2002. A triple mutant of *Escherichia coli* lacking secondary acyl chains on lipid A. J. Biol. Chem. **277:**14194–14205.
59. **Walsh, C.** 2003. Antibiotics that act on cell wall biosynthesis, p. 23–49. *In* C. Walsh (ed.), Antibiotics: actions, origins, resistance. ASM Press, Washington, DC.
60. **Weiss, D. S.** 2004. Bacterial cell division and the septal ring. Mol. Microbiol. **54:**588–597.
61. **Wiedemann, B., D. Pfeifle, I. Wiegand, and E. Janas.** 1998. Beta-lactamase induction and cell wall recycling in gram-negative bacteria. Drug Resist. Updat. **1:**223–226.
62. **Wiegand, I., K. Hilpert, and R. E. Hancock.** 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. **3:**163–175.
63. **Wiegand, I., A. K. Marr, E. B. Breidenstein, K. N. Schurek, P. Taylor, and R. E. Hancock.** 2008. Mutator genes giving rise to decreased antibiotic susceptibility in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **52:**3810–3813.
64. **Wolter, D. J., N. D. Hanson, and P. D. Lister.** 2005. AmpC and OprD are not involved in the mechanism of imipenem hypersusceptibility among *Pseudomonas aeruginosa* isolates overexpressing the *mexCD-oprJ* efflux pump. Antimicrob. Agents Chemother. **49:**4763–4766.
65. **Wood, L. F., A. J. Leech, and D. E. Ohman.** 2006. Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: Roles of sigma (AlgT) and the AlgW and Prc proteases. Mol. Microbiol. **62:**412–426.